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The Potential of Hydrolysate from Rabbit Meat Protein as an Angiotensin Converting Enzyme Inhibitor

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ABSTRACT

This research aimed to investigate the rabbit meat hydrolysate potential as an angiotensin-converting enzyme (ACE) inhibitor. Indonesian local rabbit meats were used in this study. The research was conducted in Department of Animal Product Technology, Faculty of Animal Science, Universitas Gadjah Mada, from August 2016 to February 2017. The local rabbit meats were hydrolyzed by pepsin, trypsin, and pancreatic. The obtained hydrolysates were then analyzed to identify the water-soluble protein content. The molecular weight of the hydrolysates were also confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The ACE inhibitory properties of the hydrolysates were analyzed in vitro. The results showed that pepsin, trypsin, and pancreatic hydrolysis showed a significant effect on the water-soluble protein content of rabbit meat ($p<0.05$). The water-soluble protein of rabbit meat hydrolyzed by pepsin, trypsin, and pancreatic were 9.41, 7.66, and 9.75 mg/mL respectively. The molecular weight of the rabbit meat hydrolysate were increased from 10 to 43 kDa; 17 to 43 kDa; and 10 to 43 kDa, after hydrolyzed by pepsin, trypsin, and pancreatic respectively. Furthermore, the ACE inhibitory properties (IC_{50}) of the hydrolyzed rabbit meat by pepsin, trypsin, and pancreatic were 439, 170, and 380 µg/mL, respectively. The rabbit meat hydrolysate showed a potential to be ACE inhibitor after hydrolyzed with pepsin, trypsin and pancreatic. Moreover, it also showed a promising potential to be used as bioactive components in different pharmaceutical applications. The highest ACE inhibitory capability was showed on trypsin hydrolysis with the total of 65.45% and IC_{50} 170 µg/mL ACE inhibition.

Keywords: ACE inhibitor, Pepsin, Pancreatic, Rabbit, Trypsin

Introduction

Angiotensin converting enzyme (ACE) is an important part of rennin-angiotensin system that regulates blood pressure. Angiotensin converting enzyme, a dipeptidyl carboxy peptidase (EC 3.4.15.1) is found at various tissues in the body, and it is an integral part of blood pressure and normal heart function (Shalaby *et al.,* 2006). This enzyme catalyzes the conversion of an inactive form of angiotensin I (Ang I) to a potent vasoconstrictor angiotensin II (Ang II). Therefore, ACE inhibitors are an important part of hypertension therapy.

Synthetic ACE inhibitors such as captopril, and enalapril are widely used for the treatment of cardiovascular and renal disease (Pfeffer *et al.*, 2006). However, synthetic ACE inhibitors have adverse side effects, such as allergic reactions, cough, and skin rashes (Jao *et al.,* 2012). Therefore, the development of ACE inhibitors derived from natural ingredients is necessary for future treatment and prevention of hypertension. Recently, food scientists are developing new ACE inhibitors derived from natural foods such as bovine casein (Yamada *et al.,* 2015), goat meat (Mirdhayati *et al.,* 2016), chicken breast (Sangsawad *et al.,* 2017) with the purpose of ACE inhibitors for the treatment of hypertension.

ACE inhibitory peptides are produced by using digestive enzymes and different combination of proteinases such as pepsin, trypsin, alcalase, chymotrypsin, pancreatic, and thermolysin (Bhat *et al.,* 2015). ACE inhibitory peptides have been found in enzymatic hydrolysates of many sources, such as albumen of egg (Miguel and Aleixandre, 2006), meat protein of *Bicep femoris* (Jang and Lee, 2005), myosin light chain protein of pork loin (Katayama *et al.,* 2007), muscle protein of beef (Jang *et al.,* 2008), β-*actin* protein of Kacang goat (Jamhari *et al.,* 2013b), whey protein of cheese (Jeewanthi *et*

al., 2017), and protein of porcine skin gelatin (O'Keeffe *et al.,* 2017).

Peptides derived from food proteins such as meat, egg, gelatin, and cheese were recommended for ACE inhibitors. Meat protein of rabbit has a high content of essential amino acids such as lysine, threonine, valine, isoleucine, leucine, and phenylalanine (Hernandez and Zotte, 2010). This study was to investigate the potential of rabbit meat protein hydrolysate as an ACE inhibitor. Hydrolysate of rabbit meat protein as an ACE inhibitor has never been investigated.

Materials and Methods

Preparation of pancreatic enzyme

Enzyme pancreatic was produced by method of Sigma (2003). Goat pancreas was washed and cut into small pieces. The goat pancreas was then weighed as much as 160 g, then added with 160 mL of 0.9% NaCl. The pancreas was then stirred overnight (C-MAG HS 7 IKAMAG, IKA-Werke GmbH & Co. KG, Germany). The extract was filtered to obtain a filtrate, and then filtration was conditioned at a pH of 7.5 to 8.0.

Rabbit meat preparation

Meat homogenate of rabbit was produced according to the method of Jamhari *et al.* (2013a). Rabbit meat was cut and weighed as much as 200 g with the addition of 400 mL water and was blended with a food processor (Panasonic) for 10 minutes. The extract was then homogenized for 5 minutes. Homogenate was then incubated (Memmert WNB 45, Memmert Co., Ltd., Germany) at 70°C for 30 minutes, then it was cool down by using ice.

The protein concentration of meat extract and hydrolysates

The protein concentration was analyzed by Biuret method Owasu-Apenten (2002). A total of 1 mL of homogenate was added with 4 mL of Biuret solution, the solution was allowed to stand for 30 minutes. Protein concentration was obtained by comparing the absorbance of sample and the absorbance of bovine serum albumin (BSA) at 540 nm (UV-1601PC, Shimadzu Co., Ltd., Japan).

Preparation of rabbit meat hydrolysates

The rabbit protein hydrolysates were produced is Katayama *et al.* (2003) method with a slight modification. Homogenate was divided into three groups, each group was hydrolyzed with one of protease (pepsin, trypsin, and pancreatic). Pepsin was obtained, Wako Pure Chemical Industries Ltd., Japan. Trypsin was obtained from Wako Pure Chemical Industries Ltd., Japan, and Pancreatic was prepared from goat pancreas. Each enzyme was added to 100 mL rabbit homogenate at a ratio of 1:50 (w/v). Homogenate with pepsin was adjusted to pH 2 with 1 M HCl, and homogenate with trypsin and pancreatic were adjusted to pH 7 and all homogenate were incubated in waterbath (Memmert WNB 45, Memmert Co., Ltd., Germany) at 37°C for 30 minutes. After 2 h digestion, hydrolysates by pepsin, trypsin, and pancreatic were adjusted to pH 7.5 with 1 M NaOH. Enzymatic activity was terminated by heating for 10 min at 95°C and then cool downed by using ice.

The activity of pancreatic enzyme

The activity of pancreatic was measured by Bergmeyer and Grassl (1983) method. A total of 0.25 mL of the enzyme solution was put into a test tube containing 0.75 mL of casein 1.5% and 0.125 mL Tris buffer pH 7. The solution was incubated at 37°C. for 10 min. The hydrolysis reaction was stopped by the addition of 0.75 mL Trichloroacetic acid 5%, while the blank tube was added 0.25 mL enzyme and Trichloroacetic acid 5% then incubated again at 37°C for 10 min, followed by centrifugation at 6,000 rpm (Centrifuge 5804R, Eppendorf AG, Hamburg, Germany) for 10 min. A total of 0.75 mL of supernatant was added to the reaction tube containing 2.5 mL Na_2CO_3 0.5 M then added 0.5 mL of folin and incubated at room temperature for 15 min. The incubation result was measured by a spectrophotometer at a wavelength of 578 nm (UV-1601PC, Shimadzu Co., Ltd., Japan).

Electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed following the method described by Jamhari (2013a). A total of 30 µL protein hydrolyzate protein of rabbit meat was mixed with 30 µL loading SDS buffer, then heated at 100°C for 5 min. Meat rabbit hydrolyzate was analyzed with SDS-PAGE using 12% Resolving Gel and 5% Stacking Gel. Electrophoresis was performed using AE-6530 mPAGE apparatus (ATTO, Japan). The protein bands were stained with coomassie brilliant blue (CBB) R-250, and the molecular weight of the protein was estimated using the Page Ruler Prestained Protein Marker (Thermo Scientific).

ACE Inhibitory assay

ACE inhibitory activity was determined by method of Cushman and Cheung (1971). A sample solution of protein hydrolysate at the amount of 6 µL of a particular concentration was mixed with 50 µL of 7.6 mM Hip-His-Leu solution (*Nacalai* Tesque Inc., Kyoto, Japan) containing 100 mM borate buffer (pH 8.3) and 608 mM NaCl. Before reacting with ACE, the sample was preincubated for 5 min at 37°C in a water bath. The reaction was initiated by the addition of 20 mL of 60 mU/mL ACE (Sigma-Aldrich Co., USA) dissolved in borate buffer (pH 8.3) containing 200 mM boric acid and 50 mM sodium tetraborate, and the mixture was incubated for 30 minutes at 37°C. The reaction was terminated by addition of 554 mL of 0.1 M HCl, except for the blank which had already added 554 mL of 0.1 M HCl before the incubation. The product (hippuric acid) of the reaction was extracted by addition of 1.5 mL of ethyl acetate and vigorous mixing, and then the

mixture was centrifuged at 2,500 rpm (1170 g) (Centrifuge 5804R, Eppendorf AG, Hamburg, Germany) for 15 minutes. One mL of supernatant was collected into another test tube and was dried at 100°C for 10 minutes. The test tube was cooled at room temperature for 10 minutes, and then 1 mL of 1 M NaCl was added to it. It was also stirred with a vortex mixer for 30 seconds. Each sample's absorbance was measured using a 228 nm spectrophotometer (UV-1601PC, Shimadzu Co., Ltd., Japan). The following formula was used to calculate the percentage of ACE-inhibitory activity:

$$\text{ACE-inhibitory activity (\%)} = \left(1 - \frac{\text{Absorbance of sample}}{\text{Absorbance of control}}\right) \text{x} 100$$

Statistical analysis

Data were analyzed by using one-way analysis of variance (ANOVA), and Duncan's multiple range test was used to establish the significance of different water-soluble protein. SDS-PAGE data, a specific activity of enzyme and inhibition of ACE activity were analyzed descriptively.

Result and discussion

Protein concentration

The protein concentration of rabbit meat before and after hydrolyzed by pepsin, trypsin, and pancreatic was presented in Table 1. The results showed that the different enzyme hydrolysis had a significant effect on the water-soluble protein concentration in rabbit meat ($P<0.05$). The protein concentration in rabbit meat before hydrolyzed was 7.08 mg/mL, then increased to 9.40, 7.65, and 9.73 mg/mL after hydrolyzed by pepsin, trypsin, and pancreatic, respectively. The sarcoplasmic protein is a protein that makes up water-soluble protein so its type and quantity will determine the water-soluble protein concentration (Jamhari, 2013a). The increased water-soluble protein concentration was due to the amount of formed peptide by the hydrolysis process (Tavano, 2013).

The pepsin, trypsin, and pancreatic hydrolysis showed different protein concentration levels. Pepsin is an endopeptidase enzyme that hydrolyzes peptides into amino acids without cleaving any specific bonds. According to Ryle (1970), pepsin has wide range of hydrolyzing specificity, which includes hydrophobic amino acid residues, thus resulting in a number of peptides during the process. Trypsin produces fewer protein concentrations due to its specific activity to certain amino acids. A study by Craik *et al.* (1985) showed that trypsin cuts the peptide bonds on the carboxylic side of arginine and lysine, resulting in less peptides. Pancreatic produces more protein concentrations as it consisted of several protease enzymes. Andriamihaja *et al.* (2013) reported that pancreatic is a complex enzyme secreted by pancreas and had proteolytic activity (trypsin, chymotrypsin, and elastase). The crude pancreatic enzymes used in this study showed a high specific activity, reaching 1363.87 U/mg, and produced the highest protein concentrations.

Protein confirmation by SDS-PAGE

SDS-PAGE of meat protein and hydrolyzed protein of rabbit meat by pepsin, trypsin, and pancreatic was illustrated in Figure 1. The results of the SDS-PAGE analysis of meat homogenate (rabbit meat protein) (D), meat hydrolysate with pepsin (P), meat hydrolysate with trypsin (T), and meat hydrolysate with pancreatic (Pc) showed that hydrolyzed protein of rabbit meat by pepsin, trypsin, and pancreatic enzymes produced more simple peptides than before hydrolyzed by protease enzymes. Rabbit meat protein had a molecular weight (MW) ranging from 17 to 95 kDa and was largely at a molecular weight of about 34 to 55 kDa. After hydrolyzed by pepsin, the molecular weight of rabbit meat protein ranged from 10 to 43 kDa and part of were at a molecular weight of about 34 to 43 kDa. Rabbit meat that hydrolized by trypsin had a molecular weight ranging from 17 to 43 and part of were molecular weight of 17 to 25 kDa. Hydrolyzed of rabbit meat by pancreatic enzyme had a molecular weight ranging from 10 to 43 and were largely at 17 kDa molecular weight. Rabbit meat that hydrolyzed by pepsin, trypsin and pancreatic enzyme produced simpler peptides indicated by the lack of thick bands of up bands formed by hydrolysis by using pepsin, trypsin and pancreatic. Jamhari *et al.* (2013a) reported that hydrolysis with a protease in Bali beef, Kacang goat, native chickens, and local ducks produced simpler protein band than before hydrolysisformed thick bands of lower bands.

The top bands show the size of the protein molecule is large while the low bands that are formed show the size of the protein molecule is small. Cahyarini (2014) reported that the thickness and thinness of bands formed due to the difference in the number and weight of migrating molecules, the thick band is the fixation of some bands. Bands that have greater ionic

Table 1. The concentration of protein extract meat rabbit hydrolysates at different enzyme (mg/mL).

Replication	Before hydrolysis	After hydrolysis		
		Pepsin	*Trypsin*	*Pancreatic*
1	7.12	9.39	7.64	9.73
2	7.02	9.44	7.72	9.64
3	7.09	9.40	7.61	9.78
4	6.96	9.46	7.73	9.87
5	7.00	9.37	7.57	9.75
Mean	7.04±0.07[a]	9.41±0.04[c]	7.66±0.07[b]	9.75±0.08[d]

The concentration of protein (mg/mL) significant to the activity of this enzyme by pepsin, trypsin, and pancreatic; [a-d]Means with the different letters in a same column are significantly by Duncan's multiple range test ($p<0.05$).

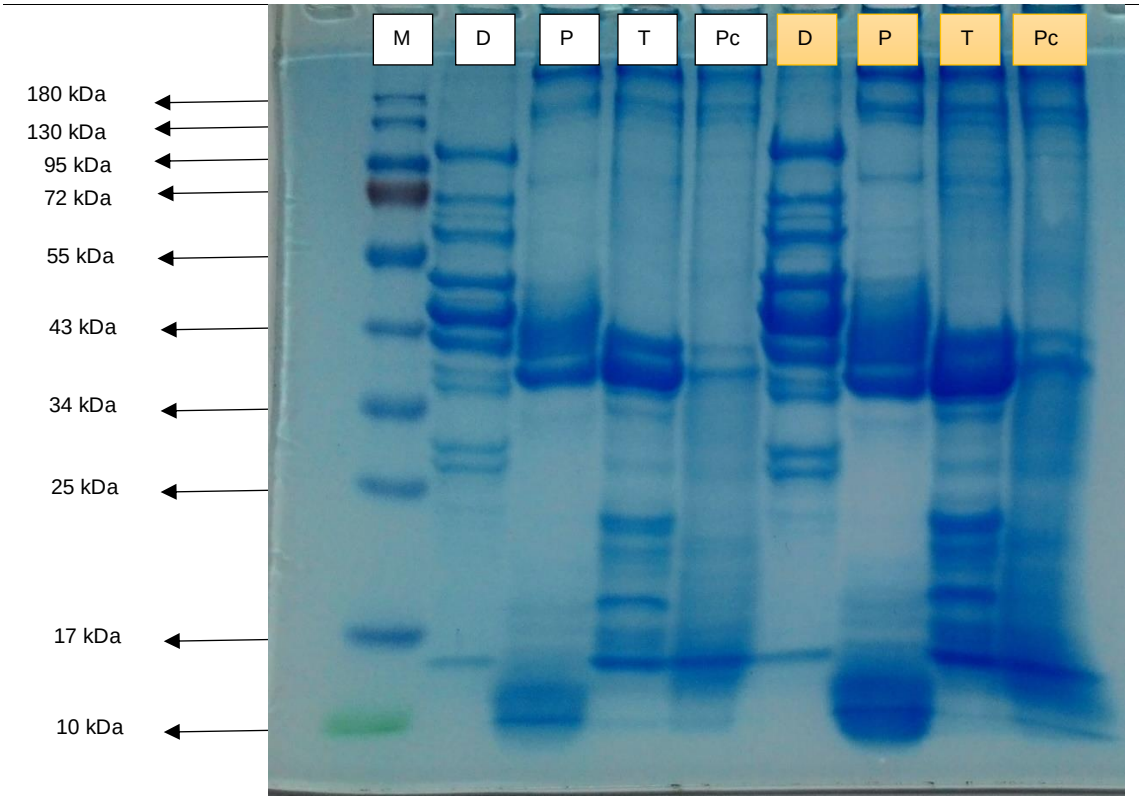

Figure 1. SDS PAGE test results of rabbit meat protein before hydrolysis and after hydrolysis, white line of the sample as much as 5 µl before hydrolysis, orange line of the sample as much as 10 µl after hydrolysis. Description: M: Marker, D: Meat before hydrolysis, P: Hydrolysis with pepsin, T: Hydrolysis with trypsin, Pc: Hydrolysis with pancreatic.

strength will migrate faster than the bands with small ionic strength.

Activity of pancreatic

Activity crude extract of pancreatic was determined using the method Bergmeyer and Grassl (1983). The working principle of the method is casein which serves as a substrate to be hydrolyzed by protease enzyme with the help of water into peptide and amino acid. The results showed that 1 mL of crude extract of pancreatic had a protein concentration of 7.07 mg/mL, the enzyme activity of 9659.72 U/mL and specific activity of crude extract of pancreatic of 1363.87 U/mg. Andriamihaja *et al.* (2013) reported that pancreatic is a complex secreted from the pancreas, which has proteolytic, amylitic and lipolytic activity. Proteolytic activity in pancreatic enzyme is divided into endopeptidase (trypsin, chymotrypsin, and elastase) and exopeptidase (carboxypeptidase A and B). The crude extract of pancreatic enzyme used in the hydrolysis of rabbit meat protein had a high specific activity, so it produced more simple peptides.

Activity of ACE-Inhibitory

Food proteins have long been recognized to have nutritional and functional properties; many studies focused on the isolation of bioactive peptides. Meat is an important source of bioactive peptides (Korhonen, 2009). The bioactive peptide has been found as an antimicrobial, antihypertensive, antioxidant, boost the immune system (Hou *et al.,* 2017). ACE inhibitor peptides have antihypertensive properties found on β-actin protein Kacang goat with IC_{50} of 120 µM (Jamhari *et al.,* 2013b) and carcass leg Kacang goat with IC_{50} of 27.0 µM (Mirdhayati *et al.,* 2016). The percentage of ACE inhibitory activity on different enzymes was shown in Figure 2.

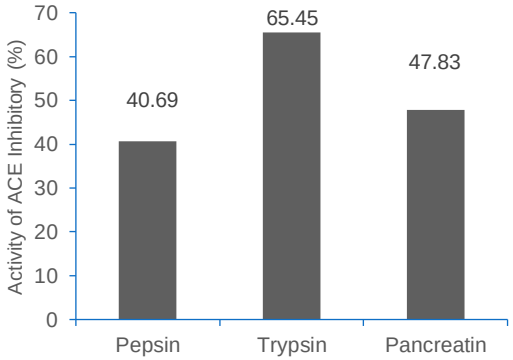

Figure 2. ACE inhibitory activity (%) hydrolysate protein of rabbit meat hydrolyzed with pepsin, trypsin, and pancreatic.

The results showed that ACE inhibitory activity of rabbit meat protein that hydrolyzed by pepsin, trypsin, and pancreatic were 40.69%, 65.45%, and 47.83%, respectively. Jamhari *et al.* (2013a) reported that hydrolyzed by pepsin,

trypsin and chymotrypsin in Kacang goat meat had an ACE inhibitory activity of 80.85%. According to our results that natural peptides that have ACE inhibitory activity have been widely identified from animal proteins that have pass through hydrolysis of protease enzymes (FitzGerald and Meisel, 2000).

The IC_{50} of hydrolyzate protein of rabbit meat taht hydrolyzed by pepsin, trypsin, and pancreatic were 439, 170 and 380 µg/mL, respectively as shown in Figure 3. The difference ACE inhibitory activity and IC_{50} were because differences the use of protease enzymes during the hydrolysis process to obtained rabbit meat protein hydrolysates thus causing differences in ACE inhibitor activity and IC_{50}. Arihara *et al.* (2001) and Muguruma *et al.* (2009), reported that the protein hydrolyzate of Bicep femoris of pigs that hydrolyzed by different enzymes produced different ACE inhibitor peptides. Pepsin has a wide specificity in hydrolyzing hydrophobic amino acid residues (Ryle, 1970). Trypsin hydrolyzes proteins into peptides specifically of certain amino acids, this scientific study of Craik *et al.* (1985) reported that trypsin cuts the peptide bonds on the carboxyl side of arginine and lysine. Pancreatic is a complex enzyme composed of trypsin, chymotrypsin, and elastase (Andriamihaja *et al.,* 2013).

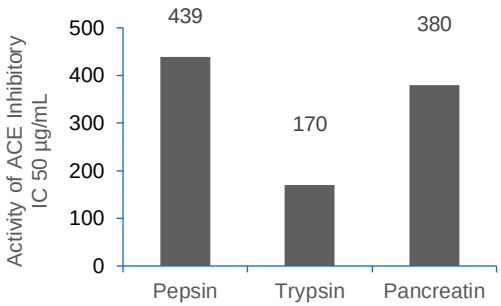

Figure 3. ACE inhibitory activity (IC_{50}) hydrolyzate protein of rabbit meat hydrolyzed with pepsin enzyme, trypsin and pancreatice.

The highest inhibitory activity was hydrolyzed by trypsin at the amount of 65.45%; this is because the peptides that hydrolyzed by trypsin had a strong affinity with the active side of ACE and can disrupt its catalytic activity thus inhibiting ACE activity in hydrolyzing Hippuril-histidyl-leucine substrate on in vitro (Ryan *et al.*, 2011).

The IC_{50} value can be defined as the amount of a protein concentration of ACE inhibitor to inhibit 50% of angiotensin converting enzyme activity. The relationship between ACE-inhibitory activity and protein concentration was illustrated in Figure 4. Then to get IC_{50} the value of Y on each equation obtained in linear regression was replaced by 50, so the value of X was IC_{50}. The IC_{50} value of the rabbit meat protein hydrolyzed by pepsin, trypsin, and pancreatic were 439, 170, and 380 µg/mL, respectively.

The majority of meat derived ACE inhibitors are grouped into true type inhibitors (Katayama *et al.,* 2004; Jang *et al.,* 2008; and Lee *et al.,* 2010). This peptide acts in two ways: first the peptide binds to the active side of the angiotensin converting enzyme or it binds to the side of the angiotensin converting enzyme inhibitor and then modifies the protein arrangement and prevents the substrate (angiotensin I) binding to the active side of the enzyme (Ryan *et al.*, 2011). ACE has an active side which is divided into three sub-sides are S1 (antepenultimate), S1' (penultimate) and S2 (ultimate) which have different characters in binding three amino acids C-terminal parts of the substrate or inhibitor, which are on two active sides homolog. As shown in Figure 5.

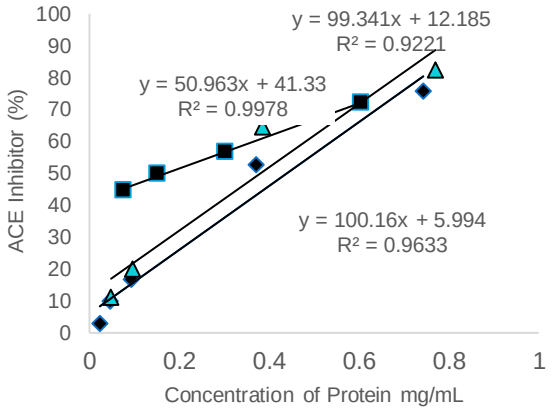

Figure 4. Graph of the relationship between the percentage of angiotensin converting enzyme and the sample protein concentration. ■ hydrolysate by trypsin, ▲hydrolysate by pancreatic, ♦ hydrolysate by pepsin.

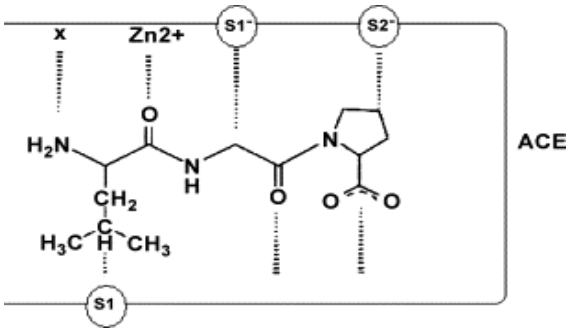

Figure 5. The interaction between the active side of angiotensin converting enzyme and angiotensin converting enzyme inhibiting peptide (Hong *et al*., 2008; Jao *et al.*, 2012).

The mechanism of action of bioactive peptides contained in meat had different with hypertension drugs in inhibiting angiotensin converting enzyme. In general, drugs block ACE and interfere with its activity, while ACE inhibitors from bioactive peptides have differently through competition with ACE. The drug works with blocking the action of ACE directly. While ACE prefers to react with ACE peptide inhibitors

without attacking angiotensin I. Inhibition of angiotensin II formation by ACE inhibitors will cause the arterial wall to relax and decrease the volume of blood fluid (Ahhmed and Muguruma, 2010).

The hydrolysates of rabbit meat protein from trypsin had a high ACE inhibitory activity of 170 µg/mL. This condition was due to rabbit meat had amino acids potentially as angiotensin converting enzyme inhibitor as reported by Hernandez and Zotte (2010) reported that rabbit meat contains such as Lysine, Threonine, Valine, Isoleucine, Leucine, and Phenylalanine amino acids. Li *et al*. (2004) reported that peptides with high ACE inhibitory activity have Tryptophan, Phenylalanine, Tyrosine or Proline residues in their C-terminal section and have branch-chain amino acids in the N-terminal section. Rabbit meat that hydrolyzed by Pepsin, Trypsin, and Pancreatic enzyme had one of the requirements of amino acids as angiotensin converting enzyme inhibitor.

Conclusions

The pepsin, trypsin, and pancreatic hydrolysis produced simpler peptides with 9.41, 7.66, and 9.75 mg/mL water-soluble protein respectively. The hydrolyzed rabbit showed an increase in molecular weight, from 10 to 43 kDa (pepsin hydrolysis), 17 to 43 kDa (trypsin hydrolysis), and 10 to 43 kDa (pancreatic hydrolysis). The result showed that rabbit meat hydrolysis by pepsin, trypsin, and pancreatic showed a potential to be utilized as ACE inhibitor and bioactive component in different pharmaceutical applications. The highest ACE inhibitory capability was showed on trypsin hydrolysis with the total of 65.45% and IC_{50} 170 µg/mL ACE inhibition.

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